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The *Plasmodium falciparum* knob-associated PfEMP3 antigen is also expressed at pre-erythrocytic stages and induces antibodies which inhibit sporozoite invasion

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Abstract

The expression of the *pfemp3* gene and the corresponding PfEMP3 knob-associated protein in the pre-erythrocytic stages of *Plasmodium falciparum* was demonstrated by RT-PCR, Western blots, IFAT and IEM. The antigen was found on the surface of the sporozoite and in the cytoplasm of mature hepatic stage parasites. Immunological cross-reactivity was observed with sporozoites from the rodent malaria parasites *Plasmodium yoelii yoelii* and *Plasmodium berghei* and was exploited to assess a potential role of this protein at the pre-erythrocytic stages. Specific antibodies from immune individuals were found to inhibit *P. yoelii yoelii* and *P. berghei* sporozoite invasion of primary hepatocyte cultures. PfEMP3 should now be added to the small list of proteins expressed at the pre-erythrocytic stages of *P. falciparum*, and its vaccine potential now deserves to be investigated.

Keywords: Gene expression; Infected hepatocyte; PfEMP3; Pre-erythrocytic; Sporozoite invasion inhibition

1. Introduction

Proteins expressed at the pre-erythrocytic stages of the life cycle of malaria parasites are involved in the acquisition of immunity against infection by the bite of infected mosquitoes. This is borne out by immunisation with irradiated sporozoites, which induces complete protection against further challenge (reviewed in Ref. [1]). The success of such immunisation depends in part on the persistence of developmentally arrested hepatic trophozoites [2–6]. Thus, proteins exposed to the host during this phase of the life cycle have been sought for inclusion in a subunit vaccine [7]. So far, two *Plasmodium falciparum* sporozoite proteins have been extensively investigated: the circumsporozoite protein (CSP) and the thrombospondin-related adhesive protein (TRAP). The protection induced following immunisation with a molecule derived from CSP was highly encouraging [8] but is yet to reach the levels obtained by radiation-attenuated sporozoites.

The difficulties in obtaining sufficient amounts of material have impeded investigations of other genes expressed at pre-erythrocytic stages. We have previously elaborated a strategy to identify such genes [7,9], which has already led to the characterisation of four new pre-erythrocytic proteins namely LSA1 [10], STARP [11], SALSA [12] and LSA3 [13]. However, these antigens only represent a subset of the 120 recombinant clones selected by this strategy, since preliminary

Abbreviations: IEM, immunoelectron microscopy; IFAT, immunofluorescence antibody test; mab, monoclonal antibody; PfEMP3, *Plasmodium falciparum* erythrocyte membrane protein 3; RT-PCR, reverse transcriptase-polymerase chain reaction..

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immunological and molecular studies indicate that the clones can be grouped in distinct sets that correspond to about 20 different genes.

We present the characterisation of two of these sets that coded for immunologically cross-reactive polypeptides. The rationale for selecting these two sets was the strong reactivity, using affinity-purified human antibodies, against *P. falciparum* sporozoites and the high prevalence of specific antibodies in sera from exposed populations. The recombinant clones were found to code for PfEMP3, an antigen previously shown to be expressed in the erythrocytic stages of the parasite [14]. We present evidence that PfEMP3 is expressed on the surface of *P. falciparum* sporozoites and in infected hepatocytes. Specific anti-PfEMP3 antibodies also strongly labelled the surface of *Plasmodium yoelii yoelii* and *Plasmodium berghei* sporozoites. This cross-reactivity was exploited to ascertain whether the protein has a functional role at the pre-erythrocytic stages. Such a role was strongly suggested by the inhibition of *P. yeolii yoelii* and *P. berghei* sporozoite invasion of primary hepatocyte cultures in the presence of specific antibodies from exposed individuals.

2. Materials and methods

2.1. Parasites

P. falciparum salivary gland sporozoites were obtained from laboratory-maintained strain NF54 [15] and from wild Thai isolates [16]. *P. falciparum* liver schizonts were identified in liver biopsies of a chimpanzee (*Pan troglodytes*) infected with the strain NF54 [17]. Intra-erythrocytic parasites (NF54 and Dd2 strains) were cultivated in vitro, as previously described [18].

2.2. Sequence analysis

Individual phage plaques carrying cloned parasite DNA were amplified by spotting on agarose plates. The insert was thereafter amplified using primers flanking the cloning site. Primers 21D (CCTGGAGCCCGTCAGTATCGGCGG) and 26 D (GGTAGCGACCGGCGCTCAGCTGG), were used at a final concentration of 0.4 μM in a reaction containing 2 mM $MgCl_2$, 0.2 mM dNTP, 1 × PCR buffer and 3.5 units of Taq polymerase (High Fidelity kit from Mannheim Boehringer, Germany). The reaction was carried out in a Crocodile III from Appligene, and initiated by heating at 94°C for 2 min before 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation for 2 min at 74°C, and terminated by a final elongation step at 72°C for 5 min. The product was purified using the Qiagen PCR Purification kit (Qiagen®, Germany), and cloned in the PCR2.1-Topo vector (Invitrogen, The Netherlands). The sequence of the insert was obtained by dideoxy sequencing with the T7 sequencing kit (Amersham Pharmacia, Sweden). Sequencing was always performed on at least three separate clones.

RT-PCR was performed with a forward primer (TTCCTTTATTCTTTAATGCTTCATCA) and a reverse primer (TATTTTTGTTTTCTCCTTTAATATGG) flanking the putative intron, following the protocol of the OneStep RT-PCR kit from Qiagen®. In order to increase the sensitivity of the assay, 1 μl of the RT-PCR product was used as template in a second PCR (35 cycles, 1 min 95°C, 1 min 58°C, 2 min 72°C, followed by a final elongation step at 72°C) with the same forward primer and a reverse primer (CCCTGAACTGCTTTTCTTTTAATGCCTCC) using the Qiagen Taq polymerase (Qiagen®, Germany). Sequence homology searches were performed using the BLAST server [19] at http://www.ncbi.nlm.nih.gov/BLAST/, against the *P. falciparum* genome databases (May 2000). The putative transmembrane domain was predicted by the HMM software [20] at http://genome.cbs.dtu.dk.

2.3. SDS–PAGE and Western Blots

SDS-extracts containing protein from equal numbers of sporozoites or blood stage parasites were subjected to electrophoresis under reducing conditions in 5% SDS–PAGE gels. Electro-blotting was performed on nitrocellulose membranes at 150 mA for 1.5 h in Tris/glycine. The membrane was probed with undiluted immunopurified antibodies or whole sera diluted 1/100. Following washing, membranes were incubated for 30 min with alkaline phosphatase-coupled anti-human or anti-rabbit IgGs (Promega) diluted 1/4000, washed three times and the colour developed by addition of the substrates NBT and BCIP (Promega).

2.4. Recombinant protein expression and purification of specific antibodies

Expression of the λgt11 recombinants (DG3, DG29f, DG64, DG114, DG222, DG400, DG662, DG693, DG1095-1) was obtained by IPTG induction [21].

The DG114 and DG662 inserts were separately subcloned in pTrcHis6 vector (Invitrogen, The Netherlands) in order to obtain His_6-tagged recombinant protein. The His_6 recombinants were purified from 1 l of bacterial culture following induction by 0.5 mM IPTG. The cells were harvested and resuspended in 25 ml of Buffer A (20 mM sodium phosphate, 8 M urea, pH 7.4), sonicated with ten 1 min bursts. The supernate obtained following centrifugation (10 000 × *g* for 10 min at 4°C), was loaded onto a 1 ml Hi-Trap Chelat-

ing-column linked with Nickel Chloride (Pharmacia) and pre-equilibrated with Buffer A supplemented with 10 mM Imidazole. The column was then washed consecutively with five volumes of Buffer A supplemented with 10, 20 and 40 mM of imidazole. The recombinant protein was eluted with 20 ml of buffer A supplemented with 100 mM imidazole, and the eluate concentrated to 5 ml and dialysed against 20 mM of L-histidine/HCl pH 6.0 overnight at 4°C. The dialysate was submitted to anion exchange chromatography on Hi-Trap Q columns from Pharmacia, which had been previously equilibrated with 20 mM of L-histidine/HCl pH 6.0. The protein was then eluted with a 20–500 mM NaCl gradient in the same buffer, and the maximal elution was observed at 200 mM NaCl.

Recombinant DG662 was subcloned into pGEX, purified as a fusion protein, and was used to immunise rabbits in order to provide specific antiserum. Antibodies against the DG662 and DG114 recombinant proteins expressed into His_6-tagged fusion protein were also obtained by immunisation of mice.

Immuno-purification of specific human antibodies contained in pooled African hyper-immune sera was carried out on induced plaque lifts of DG3, DG29f, DG64, DG114, DG222, DG400, DG662, DG693, DG1095-1, as previously described [9]. The reactivity of the purified antibodies was tested by Western blots of the recombinant proteins, and the lack of cross-reactivity was confirmed with irrelevant recombinant proteins of *P. falciparum*.

2.5. Human sera

Hyper-immune sera were obtained from individuals residing in a highly endemic area of the Ivory Coast (>50 infective bites per year). Sera were also obtained from residents in the Podor region of Senegal, an area of very low endemicity with a maximum of three infective mosquito bites per year [22]. Finally, serum was collected from two individuals who accidentally contracted *P. falciparum* malaria following transfusion with infected blood. It is noteworthy that one of the patients harboured parasites for a total of 45 days, the parasitaemia having reached 26% when the correct diagnosis was made and treatment successfully carried out. Serum samples were also obtained from three volunteers immunised by irradiated *P. falciparum* sporozoites and protected against a viable challenge (a gift from David Haynes, WRAIR, Bethesda, MD, and from Robert Edelman, from the CVD, Maryland University, Baltimore, MD).

2.6. Indirect fluorescence antibody test

Surface immunofluorescence was performed on sporozoites deposited on poly-L-lysine coated slides in a 'wet' sporozoite assay [23]. For the liver stages, both Carnoy-fixed material and 5 μm cryo-sections from unfixed material stored in liquid nitrogen were used [24]. Acetone-fixed thin blood smears from synchronised *P. falciparum* blood cultures in ring and schizont stages were used in a standard IFAT assay using as a second antibody a fluorescein-labelled anti-IgG + M (Diagnostics Pasteur) diluted 1/200 in PBS.

2.7. Immunoelectron microscopy

This was carried out as previously described [25]. Briefly, *P. falciparum* salivary glands were fixed in 0.1% glutaraldehyde, 0.1% formaldehyde and embedded in LR gold Resin (Poly Science Inc, Warrington, PA). Sections were incubated with eluted human antibodies against DG662, DG64 or DG114 and subsequently with goat antihuman IgG conjugated to gold particles (15 nm in diameter, Amersham). Liver sections were fixed in 2% glutaraldehyde, stained with uranyl acetate and lead citrate, and examined in a JEOL 100CX electron microscope.

2.8. Inhibition of sporozoite invasion (ISI)

Human antibodies affinity purified on the recombinant proteins DG114 and DG222 were tested for their inhibitory effect in an assay of *P. yoelii yoelii* invasion into hepatocytes, as previously described [26]. Briefly, 10^5 mouse hepatocytes/chamber (suspended in a complete medium) were seeded in an eight-chamber Lab-Tek plastic slides (Nunc Inc., Napper Ville, IL). After an incubation of 24 h at 37°C under a 5% CO_2 atmosphere, the medium was removed, and anti-DG114 or anti-DG222 antibodies were added simultaneously with 6×10^4 *P. yoelii yoelii* (clone 1.1) or *P. berghei* (ANKA strain) sporozoites. NYS1, an IgG3 monoclonal antibody directed against *P. yoelii* CSP [27], was used to provide a control for the assay. After 3 h at 37°C, the medium containing sporozoites and antibodies was discarded and replaced by normal supplemented medium. After an additional 48 h, the hepatocytes were fixed for 10 min in cold methanol, and developing *P. yoelii yoelii* liver stages were counted following identification by IFAT with the mab NYLS3 [28]. Parallel experiments were performed with *P. berghei* to demonstrate the cross-species-specific inhibition. The mab 3D11 directed against *P. berghei* CSP was used as control and to detect liver schizonts by IFAT.

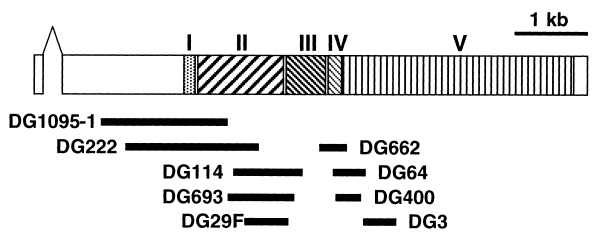
The total number of liver schizonts in each culture well was counted by epifluorescence using an Olympus ultra-violet microscope, and the mean number of liver schizonts were calculated in duplicate culture wells. Results were expressed as the percentage of inhibition, calculated as: $(\text{Nu-Nl}/\text{Nu}) \times 100$

(Where Nu = number of liver schizonts in untreated cultures; and Nl = number of liver schizonts in test cultures).

3. Results

3.1. Assignment of cloned inserts to pfemp3

Screening of a *P. falciparum* T9-96 genomic library with serum from an individual exposed to sporozoite challenge but not to erythrocytic parasites resulted in the isolation of 120 positive clones [9]. Human antibodies from hyperimmune individuals were affinity-purified using individual recombinant proteins and tested by IFAT against *P. falciparum* sporozoites. Antibodies against two recombinant proteins (DG114 and DG662) that displayed a particularly strong IFAT labelling were then used to screen for other immunologically cross-reactive recombinant proteins. Four such clones were found for the DG114-specific antibodies and three for the DG662-specific antibodies (Fig. 1). Sequencing revealed that, within the two independent sets of clones, inserts consisted of overlapping fragments. A database search assigned these two sets of clones to two non-overlapping regions of a single *P. falciparum* gene (Fig. 1A), namely *pfemp3* [14], which is located on chromosome 2 [29]. These two groups of recombinant proteins encompass two separate B-cell epitope regions on this gene, which were denoted as the N-region and the C-region. The recombinant proteins expressed by clones DG114 and DG222 were later used as being representative of the N-region, and those of DG64 and DG662 of the C-region.



	N-region				C-region			
	r222	r114	r693	r29F	r662	r64	r400	r3
e222	++	++	+	-	-	-	-	-
e114	++	++	++	++	+	-	-	-
e693	++	++	++	++	+	-	-	-
e29F	-	++	++	++	-	-	-	-
e662	-	-	-	-	++	++	++	++
e64	-	-	-	-	++	++	++	++
e400	-	-	-	-	++	++	++	++
e3	-	-	-	-	++	++	++	++

Fig. 1. Schematic representation of *pfemp3* (clone 3D7), with the name and the position of the cloned inserts presented underneath (to scale). The repeat regions are labelled I–V, and the sequence of the repeat units of regions III and IV are closely related. Repeat region V consists of 81 × 13-mer units. Immunological cross-reactivity data obtained by Western dot blotting are presented in Table 1. The recombinant proteins (prefix 'r') were used to elute antibodies from hyperimmune sera (prefix "e"). + +: strong reactivity, +: reactivity, −: no reactivity.

3.2. Expression of pfemp3 in pre-erythrocytic stages

The expression of *pfemp3* in sporozoites and infected hepatocytes was investigated immunologically and genetically. Transcription of *pfemp3* was assessed by RT-PCR assays using total RNA extracted from sporozoites or from blood-stage parasites (rings or schizonts). Amplification was directed against the fragment including the putative intron at the 5′-end of the gene. A PCR product was observed for both sporozoites and blood-stage parasites (Fig. 2A). The size of the fragment obtained by RT-PCR was smaller than that obtained by amplification of genomic DNA, thus demonstrating that splicing of the intron occurs in both erythrocytic and exoerythrocytic stages of the life cycle. The predicted splice site [29] was confirmed by sequencing of the RT-PCR products and was found to be the same in the RNA obtained from sporozoites and blood stage parasites. Synthesis of the protein in these two stages was then confirmed by Western blot analysis, IFAT and IEM.

Western blots of protein extracts from sporozoites and red blood cells infected with *P. falciparum* were probed with individual pools of antibodies specific to each of the following recombinant proteins: DG222, which spans the N-terminus domain and the repeat regions I and II, DG3 (part of repeat region V) and DG662 (part of repeat region III, all of IV), which are located in the C-region (Fig. 1). A representative result of each is presented in Fig. 2B–C. For blood schizonts, N-region specific antibodies affinity-purified from hyperimmune sera recognised a single polypeptide of 319 kDa, corresponding in size to that previously described for PfEMP3 [14,30]. In blots probed with sera from a rabbit immunised with the recombinant protein from DG662 (C-region), three bands of 319, 280 and 250 kDa were observed in the ring lane, and only the 250 kDa bands was observed in the schizont lane (Fig. 2C). It is likely that the two lower-molecular-mass polypeptides are proteolytic products derived from the 319 kDa PfEMP3. Western blots of sporozoite extracts showed a similar pattern of reactivity, namely the presence of a single 319 kDa polypeptide when probed with antibodies directed against the N-region, whilst for antibodies directed against the C-region, this band was faintly

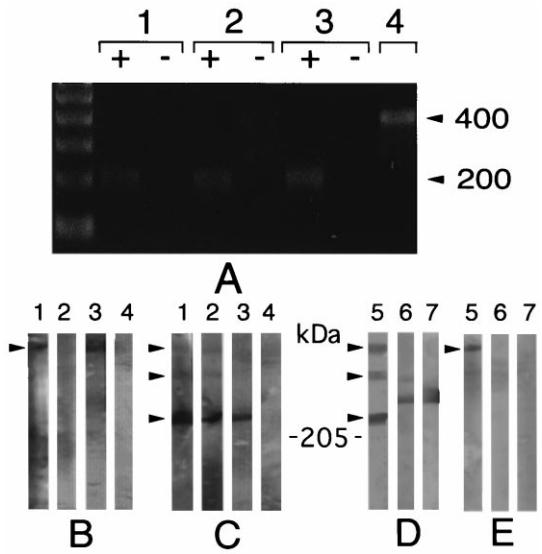


Fig. 2. (A) Product amplified by RT-PCR of total RNA purified from *P. falciparum* NF54 strain in sporozoite stage (lane 1), blood-stage ring forms (lane 2) and blood-stage schizont forms (lane 3) with (+) and without (−) RT-PCR. The product obtained using the same set of primers with genomic DNA (NF54) as a template is shown in lane 4. Western Blots of *P. falciparum* protein extracts probed with human antibodies affinity-purified against DG222 corresponding to the N-region (B), and antibodies raised in a rabbit immunised with DG662 corresponding to the C-region (C). The lanes were loaded with protein extracts obtained from similar numbers of parasites: 10^6 sporozoites (lanes 1), 10^6 blood stage ring forms (lanes 2), and 10^6 blood schizonts (lanes 3). Non-parasitised red blood cells were loaded in lanes 4. Similar blots probed with antibodies specific to different recombinant proteins corresponding to the two regions showed the same pattern. Protein extracts from two different parasite lines were also probed with mouse antibodies raised against recombinant proteins corresponding to the N- and the C-regions of PfEMP3 (DG114 for the lanes in D, and DG662 for the lanes in E). The lanes were loaded with protein extracts obtained from similar numbers of parasites: 10^6 schizonts from the NF54 strain (lanes 5), 10^6 from the Dd2 cloned line (lanes 6) and uninfected red blood cells (lanes 7).

labelled, but the 280 kDa band and a 250 kDa band were strongly labelled (Fig. 2B–C). It should be noted that protein degradation is frequently observed when extracts are made from sporozoites. In order to confirm that the protein(s) identified do correspond to PfEMP3, a Western blot of extracts from blood stage parasites obtained from Dd2, a parasite line lacking the *pfemp3* gene, was probed with mouse anti-sera raised against another N-region recombinant (DG114) and DG662 the C-region recombinant (Fig. 2D and E). For the two anti-sera, no specific bands were observed for the Dd2 parasite, which lacks the *pfemp3* gene (lanes 6), whereas the pattern obtained with the positive control NF54 (lanes 5) was similar to that observed for the human and rabbit sera.

Having confirmed that the affinity-purified antibodies react specifically with PfEMP3 in both erythrocytic parasites and sporozoites, the localisation of PfEMP3 in pre-erythrocytic stages was determined through IFAT and IEM studies using *P. falciparum* sporozoites and liver forms. The antibodies were tested by IFAT against blood-stage parasites (rings and schizonts), and resulted in a fluorescence pattern (Fig. 3C) that was consistent with the described location of the antigen at the membrane of the infected red blood cell [14,31]. At the sporozoite surface, labelling was homogeneous and very intense (Fig. 3A). A similar pattern was observed by IEM, where large numbers of gold particles were specifically retained on the surface of sporozoites, with a suggestion of clustering (Fig. 3E). In sub-mature 5-day-old liver forms, the antigen was mainly detectable by IFAT in the cytoplasm of the parasite (data not shown), but was equally distributed at the periphery in mature 6-day-old liver schizonts (Fig. 3B). This was confirmed by IEM of these liver schizonts where gold

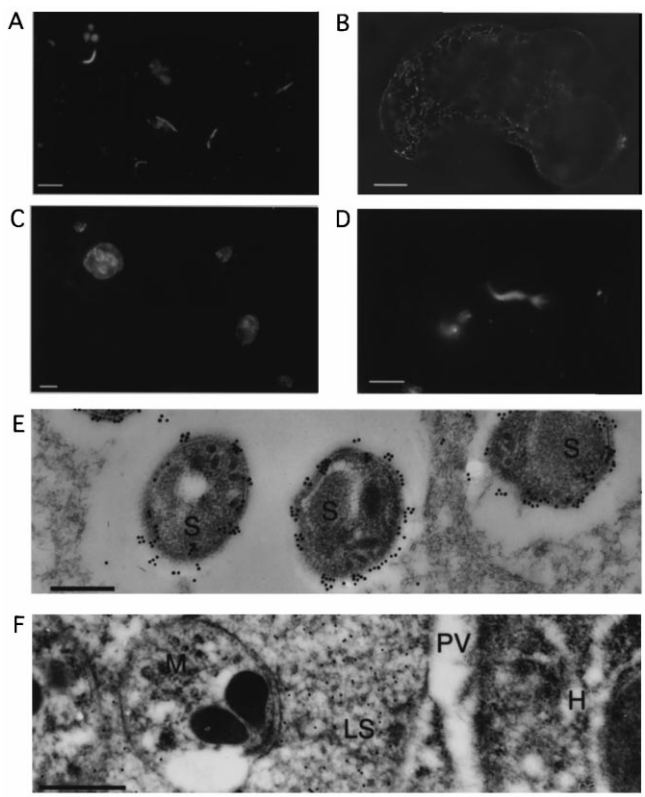


Fig. 3. IFAT labelling of *P. falciparum* sporozoites (A), 6-day-old liver schizont (B), blood-stage parasites (C) showing two rings and one schizont; sporozoites of *P. yoelii yoelii* are shown in (D). Bars in (A)–(D) represent 10 μm. IFAT was performed using unfixed NF54 sporozoites labelled with anti-DG114 antibodies. (E) IEM showing cross-sections of *P. falciparum* sporozoites, labelled S, showing deposition of gold particles at the surface of the sporozoites. (F) IEM showing that gold particles are evenly deposited in the cytoplasm of the liver schizont, LS, and are only sporadically seen over the merozoites, M, seen at the parasitophorous vacuole, PV, or in the host cell cytoplasm, H. Bars in (E)–(F) represent 1 μm. Sections for IEM were stained with human affinity-purified anti-DG662 antibodies. It should be noted that the gold particles used for the sporozoite sections were larger (30 nm) than those used for the liver schizont sections (10 nm).

particles were distinctly confined to the parasite cytoplasm, whereas the flocculent material released from the parasitophorous vacuole remained unlabelled (Fig. 3F). This pattern of reactivity was observed irrespective of the antibodies used (N-region or C-region specific), and only representative examples are presented.

Finally, anti-PfEMP3 antibodies were detected, by ELISA against the DG64 recombinant protein, in the sera of three volunteers immunised with *P. falciparum* irradiated sporozoites, which were therefore exposed to pre-erythrocytic but not to blood-stage parasites. However, one of two transfusion malaria sera, was found to be negative with this recombinant protein despite the long duration and high-grade parasitaemia.

3.3. Conservation of PfEMP3 epitopes in P. falciparum

In order to evaluate antigenic conservation of PfEMP3 amongst different *P. falciparum* isolates, surface IFATs were performed on sporozoites obtained by feeding *Anopheles dirus* on gametocytes from seven Thai isolates. Antibodies specific to the N-region reacted with all seven isolates, whereas anti C-region antibodies reacted with only five out of seven. In all positive cases, all the sporozoites, which may have originated from genetically heterogeneous populations, were labelled. The antigenic conservation of PfEMP3 was also evaluated indirectly by measuring the prevalence of antibody responses in sera from defined human groups from two geographical regions of varying levels of malaria endemicity. The prevalence of naturally acquired antibody responses to PfEMP3 was determined by Western dot blots of recombinant proteins corresponding to the N- or C-region. Seventeen of the 18 sera collected from individuals living in a high transmission area reacted with N-region recombinant proteins, 15 reacted with C-region recombinant proteins, and only one serum failed to react. Sera from 43 individuals residing in a very low transmission area, were then tested against a recombinant protein from the C-region (DG64). The prevalence of specific antibodies increased as a function of age, and hence exposure, with 33, 55 and 100% of the sera testing positive in subjects <10, 10–20, and >20 years of age, respectively. It is interesting to note that the age-dependent prevalence of antibodies to total blood stage antigens measured by IFAT using the same sera is 66, 100 and 100% for the same age groups, respectively. This indicated that the acquisition of anti-PfEMP3 antibodies is not necessarily correlated with exposure to blood-stage parasites.

3.4. Cross-reactivity with other species

In order to determine whether a protein cross-reactive with PfEMP3 occurs in other malaria species, the same set of affinity-purified antibodies was tested by IFAT on sporozoites of *Plasmodium vivax* and on those of two murine malaria species *P. yoelii yoelii* and *P. berghei*. In *P. vivax*, only antibodies directed to the C-region could be tested, and these were found to be positive (data not shown). Cross-reactivity was reproducibly observed for the sporozoites of the two rodent malaria species, using antibodies directed against various recombinant proteins of the N-region of PfEMP3, which were specific for PfEMP3 as they detected a single band in Western blots of total parasite extracts. The immunofluorescence pattern was similar to that observed for *P. falciparum* sporozoites, in that both the *P. y. yoelii* and *P. berghei* sporozoite surface was intensely and homogeneously labelled (Fig. 3D).

3.5. Biological effect of anti-PfEMP3 antibodies

Functional studies of the PfEMP3 on *P. falciparum* pre-erythrocytic stages are highly restricted by the simultaneous requirement of viable *P. falciparum* sporozoites as well as primary human hepatocyte cultures. We took advantage of the strong cross-reactivity with a sporozoite antigen from the murine species *P. berghei* and *P. yoelii yoelii*, for which the above limitations are relatively easier to overcome, in order to investigate a potential effect of naturally acquired human anti-PfEMP3 antibodies directed against the N-region, which are consistently found to react with the surface of sporozoites, on the in-vitro invasion of primary mouse hepatocytes by *P. berghei* or *P. yoelii yoelii* sporozoites. Human antibodies affinity-purified from a pool of hyperimmune sera using the recombinant proteins DG222 and DG114 were found to inhibit *P. yoelii yoelii* sporozoite invasion by 95 and 88%, respectively (Table 1). The results were duplicated using different batches of immunopurified antibodies, sporozoites and liver cells. A similar level of invasion inhibition (77%) was obtained using anti-DG222 antibodies in tests performed using *P. berghei* sporozoites.

4. Discussion

PfEMP3 was first described in blood-stage *P. falciparum* parasites where its association with knobs at the membrane of the infected red blood cell suggested that it might play a role in cytoadherence [14]. In this article, we present evidence that PfEMP3 is also expressed by pre-erythrocytic stage parasites, namely the sporozoite and the infected hepatocyte. The inhibition of *P. yoelii yoelii* sporozoite invasion by human antibodies against PfEMP3 further suggests that this antigen has a functional role during the sporozoite stage and might be a natural target for protective mechanisms.

Table 1
Sporozoite invasion inhibition assays

Antibodies	*P. yoelii yoelii*		*P. berghei*	
	Number of schizonts[a]	Percentage inhibition[b]	Number of schizonts	Percentage inhibition
Anti-DG222	9/0	95.5	26	77
Anti-DG114 (1/10)	10/16	88.1	nd	nd
Anti-DG114 (1/100)	24/20	79.9	nd	nd
No antibody	100/119	0	108	0
mab NYS1 anti-*P. yoelii yoelii* CS	164/168	−51[c]	138	−28[c]
mab 3D11 anti-*P. berghei* CS	82	31	30	74

[a] Results from two duplicate assays.
[b] Arithmetic mean of percentage inhibition.
[c] Enhancement, rather than inhibition, of invasion has already been reported at the anti-*P. yoelii yoelii* CS monoclonal antibodies concentration that we have used [37].

Clones expressing PfEMP3 were found amongst a subset of plaques of an λgt11 expression library of *P. falciparum* (T9-96). This subset of 120 plaques was selected using antibodies present in the serum of an individual who was exposed to infective mosquito bites but not to blood-stage parasites [9]. The main criteria for selection were the presence of specific antibodies against the recombinant protein in the sera from immune persons and their recognition of *P. falciparum* pre-erythrocytic stages. So far, four new antigens expressed during pre-erythrocytic stages have been brought to light using this approach. Nine clones expressing various fragments of PfEMP3 were obtained from this subset of 120 recombinant phages. The selected nine clones could be classified into two clusters, the first made up of five overlapping fragments corresponding to the N-terminal half of PfEMP3, and the other consisting of four overlapping fragments located at the C-terminal region of this protein, thus corroborating the antigenic cross-reactivity observed in preliminary experiments. The complete sequence of *pfemp3* is found in the fully sequenced chromosome 2 of the 3D7 clone of *P. falciparum* [29]. The gene consists of a 111 bp mini-exon, followed by a putative 195 bp intron and a large second exon (7215 bp), which consists of two small conserved regions flanking four blocks of repeats.

Evidence for the expression of PfEMP3 at the pre-erythrocytic stages was derived from a number of different approaches. RT-PCR analysis resulted in the amplification of a fragment of similar size from total RNA purified from *P. falciparum* sporozoites or from cultured blood-stage parasites. Sequencing of the product which was smaller, by 195 bp, than that obtained from amplification using genomic DNA, confirmed the predicted splicing site of the RNA and indicates that splicing occurs in both sporozoites and blood-stage. Although the sensitivity of PCR might detect RNA from spurious 'leaky' transcription, this RNA is rarely spliced. It is therefore likely that the spliced mRNA detected in the sporozoites is functional. Western blotting of protein extracts from both sporozoites and blood-stage parasites showed the intact PfEMP3 protein and the same degradation products in both stages. Analysis by IFAT using specific affinity-purified antibodies resulted in a strong labelling at the surface of the sporozoites. With erythrocytic stages, IFAT labelling was similar to that observed previously [14,32], in that labelling was only seen in the cytoplasm and the parasitophorous vacuole of rings, but it extended to the membrane of the infected red blood cell in the mature schizont. For the liver stages, the antigen was mainly confined to the cytoplasm in contrast to most other pre-erythrocytic antigens studied to date which tend to segregate to the parasitophorous vacuole [7]. The IFAT results observed for both pre-erythrocytic stages were further confirmed by IEM. The possibility that the specific antibodies used actually recognise a cross-reactive protein in the two stages rather than, or in addition to, PfEMP3 is difficult to reconcile with the data we present: first, antibodies to non-cross-reactive epitopes present at each end of this large protein produce the same Western blotting and IFAT patterns, and second, no bands or IFAT labelling are observed when the *P. falciparum* Dd2 line, which lacks the *pfemp3* gene, is used.

These observations at pre-erythrocytic stages are consistent with the observed transport of the PfEMP3 in asexual blood stages [33]. The export of PfEMP3 to the surface of the parasite is consistent with the mini exon that codes for a 37 aa putative signal peptide, a feature that is shared by a number of other membrane-targeted proteins [11,34]. This transport occurs via small vesicles where PfEMP3 co-localises with PfEMP1, and recently, gene-disruption experiments targeting PfEMP3 suggested that the efficiency of PfEMP1 transport depends on the expression of the complete *pfemp3* gene [35]. Our data strongly suggest that in sporozoites, the PfEMP3 is found on the outer side of the parasite limiting membrane. This contrasts with the location of this antigen either in the parasite cytoplasm or below the

membrane of the infected red blood cell. Export of this antigen to the surface of the infected hepatocyte cannot be excluded since a negative IFAT result does not imply a total absence, and only small amounts could be sufficient for efficient presentation by MHC molecules. Whether PfEMP3 associates with other molecules in sporozoites and liver stage parasites, as described in blood stages, remains to be investigated. It is none the less highly interesting that PfEMP3 appears to be directly exposed to the immune system only in sporozoites, but retains an intracellular location throughout the other stages of the life cycle in the mammalian host.

Although antibodies directed against PfEMP3 are prevalent in the blood of persons living in malaria endemic areas, this immune response can result equally from exposure to the antigen expressed in the blood stages as in the pre-erythrocytic stages. The presence of specific anti-PfEMP3 antibodies in the serum of three persons solely exposed to irradiated or viable *P. falciparum* sporozoites strongly indicated that PfEMP3 is expressed in pre-erythrocytic parasites and in sufficient amounts to generate a specific immune response. Conversely, the absence of immune response in a transfusion malaria patient harbouring a parasitaemia of up to 26% for 45 days suggests either that it may not be consistently immunogenic at blood stage level or, more likely, that immune responses might be preferentially targeted to polymorphic regions. Although data on the prevalence of natural antibodies against PfEMP3 suggest that the protein does not display any major antigenic diversity, it is possible that the C-region displays some polymorphism since antibodies directed against this region failed to label some isolates by IFAT. This might be due to the fact that the large block of repeats of the C-region made up of repeat units displays a higher degeneracy at the amino acid level, as compared to those in the N-region.

The interspecies cross-reactivity of PfEMP3 was striking. This cross-reactivity was observed using antibodies directed against the N-region that recognise a single band in Western blots of *P. falciparum* blood-stage proteins, i.e. do not display cross-reactivity with other proteins. This suggests that PfEMP3 homologues might be present in *P. vivax*, *P. berghei* and *P. yoelii yoelii*, thus underlying the functional importance of this protein. The strong cross-reactivity with *P. berghei* and *P. yoelii yoelii* sporozoites was exploited to provide evidence that PfEMP3 might indeed be associated with an important biological role during sporozoite invasion. The high levels of inhibition of sporozoite invasion into primary mouse hepatocytes observed using affinity-purified specific human antibodies compares favourably with those obtained in inhibition assays of *P. falciparum* sporozoite invasion of primary human hepatocytes by antibodies against the CS and STARP antigens from this species [36].

The expression of PfEMP3 on sporozoite surface, in liver stages and asexual blood stages, and the potential existence of homologous antigens in other species suggest that PfEMP3 is likely to play a major role throughout the life cycle of *Plasmodium* parasites. The ability of PfEMP3 to associate with the parasite and/or the host cell membrane is a feature common to all stages. It is noteworthy that a substantial proportion of the phage clones selected by our original screening of the *P. falciparum* expression library (9/120) have been shown to belong to *pfemp3*. Furthermore, the affinity-purified antibodies against the PfEMP3 recombinant proteins displayed the highest reactivity observed so far. The strong IFAT and IEM labelling of sporozoites suggests that the protein is abundant at the surface of the sporozoite, where it induces antibodies capable of inhibiting sporozoite invasion. Whether PfEMP3 associates with MHC molecules at the surface of infected hepatocytes, and can thus constitute a specific T-cell target, remains to be determined. In conclusion, PfEMP3 can now be added to the small list of proteins known to be expressed at the pre-erythrocytic stages of *P. falciparum*; moreover, the direct exposure of PfEMP3 on sporozoites to the immune system would make it another candidate for inclusion in sub-unit vaccines against these stages.

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